Assessment Run 74 2025

BRAF (BRAF V600E)

Purpose
Evaluation of the technical performance and level of analytical sensitivity and specificity of BRAF IHC tests among NordiQC participants for the demonstration of corresponding BRAF V600E mutations in melanomas and colorectal adenocarcinomas.

Material
The slide to be stained for BRAF comprised:

1. Appendix, 2. Malignant melanoma without BRAF V600E mutation*, 3. Malignant melanoma with BRAF V600E mutation*, 4. Colon adenocarcinoma without BRAF V600E mutation*, 5. Colon adenocarcinoma with BRAF V600E mutation*.

*BRAF V600E mutation status confirmed by Next Generation Sequencing (NGS).

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a BRAF staining as optimal included:
- An at least weak to strong distinct unequivocal cytoplasmic staining reaction in virtually all neoplastic cells in the malignant melanoma tissue core no. 3 and the colon adenocarcinoma tissue core no. 5.
- No staining reaction in neoplastic cells in the malignant melanoma tissue core no. 2 and the colon adenocarcinoma tissue core no. 4.
- No or only faint cytoplasmic staining reaction in epithelial cells in appendix. A weak staining reaction in the smooth muscle layer and nuclear staining reaction in epithelial cells was accepted.

KEY POINTS FOR BRAF V600E IMMUNOASSAYS
- A high overall pass rate of 81% was observed in this 2' assessment focusing on BRAF V600E mutations in colon adenocarcinomas and melanomas.
- The mAb clone **VE1** was used by 91% of all participants.
- The VE1 **RTU** system 760-5095 from Ventana/Roche applied by vendor recommended protocol settings was most successful giving pass rates of 97%.
- The performance of the Ventana/Roche VE1 RTU system 760-5095 was also found successful when optimized by using OptiView with amplification.

Participation

Number of laboratories registered for BRAF, run 74	238
Number of laboratories returning slides	211 (89%)

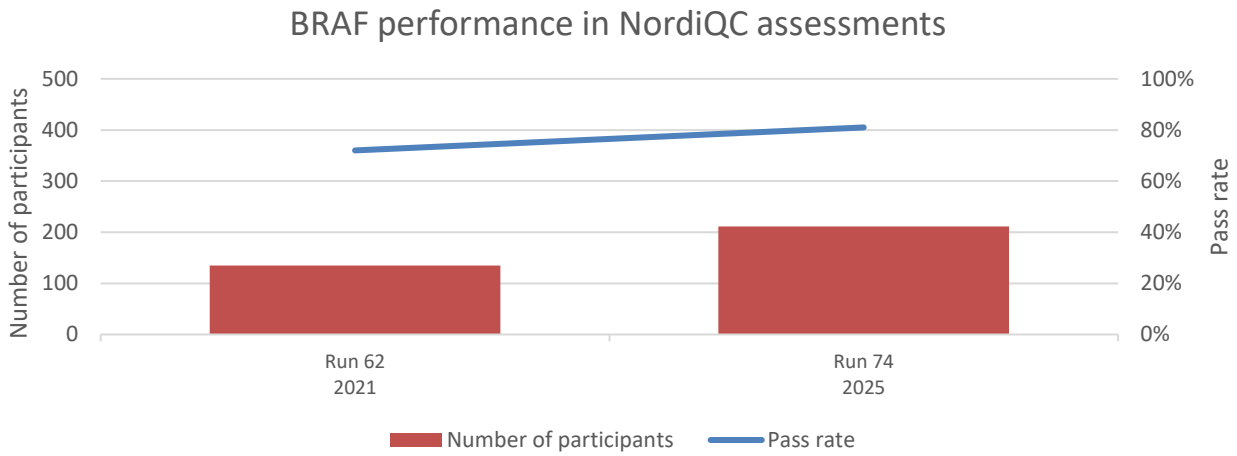
Results
At the date of assessment, 89% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

211 laboratories participated in this assessment and 81% achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 3).

The most frequent causes of insufficient staining reactions were:
- Less successful primary antibodies
- Insufficient HIER - too short HIER time
- Too long incubation of primary Ab
- Too high concentration of primary Ab

Performance history
This was the second NordiQC assessment of BRAF. An increase in pass rate was observed compared to the previous run (see Graph 1), which primarily is due to the use of robust primary antibodies and well calibrated Ready-To-Use (RTU) systems (see Table 1).

Graph 1. **Proportion of sufficient results for BRAF in the second NordiQC runs performed**



Control
Tumors confirmed with and without BRAF V600E mutation are recommended as positive and negative tissue controls for BRAF. Appendix can also serve as a negative tissue control, where no or only a faint cytoplasmic staining reaction should be seen in the epithelial cells. At present, no data is available on consistent low-level expressing normal tissues/cells, and thus it is important to secure a distinct and an "as strong as possible reaction" for BRAF in mutated tumors and still no reaction in negative tissue controls.

Conclusion
In this second NordiQC assessment of BRAF, optimal staining results could be obtained with the mouse monoclonal Ab (mAb) clones **IHC600**, **MX125**, **VE1** and the rabbit monoclonal (rmAb) clone **135K5D1** providing HIER being applied in an alkaline buffer.
The widely used mAb clone VE1 provided the highest amounts of optimal results, particularly when used as a RTU system on Ventana Benchmark platforms.
The Ventana/Roche VE1 RTU system provided the highest pass rate by vendor-recommended protocol settings (97%), but also by modifications using OptiView with amplification giving a more binary staining result. However, protocols with too high analytical sensitivity often caused false positive results interfering with the interpretation.
The corresponding concentrated format of mAb clone VE1 showed an overall reduced performance, especially on the Leica Biosystems Bond platforms typically giving aberrant nuclear reaction and false positive staining in the normal epithelium of the appendix. On the Ventana/Roche and Dako/Agilent platforms attention to proper antibody concentration, retrieval duration, and amplification settings were vital for avoiding results like weak signals or background staining.

Table 1a. **Overall results for BRAF, run 74**

	n	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
Concentrated antibodies	40	15	7	10	8	55%	38%
Ready-To-Use antibodies	171	103	45	18	5	87%	60%
Total	211	118	52	28	13		
Proportion		56%	25%	13%	6%	81%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of Optimal Results.

Table 1b. **Concentrated antibodies and assessment marks for BRAF, run 74**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb **VE1**	28 1	Abcam Spring Bioscience	14	6	6	3	69%	48%
mAb **IHC600**	5 2	GenomeME Dianova	1	1	4	1	29%	14%
rmAb **RM8**	3	Biosciences	-	-	-	3	-	-
rmAb **ZR6**	1	Zeta Corporation	-	-	-	1	-	-
Total	40		15	7	10	8		
Proportion			38%	17%	25%	20%	55%	

1) Proportion of sufficient stains (optimal or good). (≥5 asessed protocols).
2) Proportion of Optimal Results.

Table 1c. **Ready-To-Use antibodies and assessment marks for BRAF, run 74**

Ready-To-Use antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **VE1 760-5095(VRPS)[3]**	33	Ventana/Roche	23	9	1	-	97%	70%
mAb clone **VE1 (163) 760-5095 (LMPS)[4]**	123	Ventana/Roche	73	34	14	2	87%	59%
mAb clone **VE1 AVI3248**	6	Biocare Medical	3	1	1	1	67%	50%
mAb clone **IHC600 V600E**	3	GenemeMe	-	1	2	-	-	-
mAb clone **IHC600 AB-138191**	1	Dianova	1	-	-	-	-	-
mAb clone **MX125 MAB-1078**	1	Fuzhou Maixin	1	-	-	-	-	-
mAb clone **V600E/1321 AMD49GP**	1	BioGenex	-	-	-	1	-	-
mAb clone **VE1**	1	Celnovte	1	-	-	-	-	-
rmAb clone **RM8 BSB 2824**	1	BioSB	-	-	-	1	-	-
rmAb Clone **135K5D1 PA416**	1	Abcarta	1	-	-	-	-	-
Total	171		103	45	18	5		
Proportion			60%	26%	11%	3%	86%	

1) Proportion of sufficient results (optimal or good). (≥5 asessed protocols).
2) Proportion of Optimal Results (OR).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 assessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(s), non-validated semi/fully automatic systems or used manually (≥5 assessed protocols)

Detailed analysis of BRAF, Run 74
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **VE1**: Protocols with optimal results were typically based on Heat Induced Epitope Retrieval (HIER) using Cell Conditioning 1 (CC1, Ventana/Roche) (7/11)* or Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako/Agilent) (7/13) as retrieval buffer. The mAb was typically diluted in the range of 1:200-1,000 depending on the total sensitivity of the protocol employed. Using these protocol settings, 17/22 (77%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

Table 2. **Proportion of optimal results for BRAF for the most commonly used antibody as concentrate on the four main IHC systems***

Concentrated antibody	**Dako/Agilent Autostainer[1]**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark[2]**		**Leica Biosystems Bond[3]**	
	TRS pH 9.0	TRS pH 6.1	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	BERS2 pH 9.0	BERS1 pH 6.0
mAb clone **VE1**	1/2**	-	6/11 (55%)	-	7/11 (64%)	-	0/5	-

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective systems.
** (number of optimal results/number of laboratories using this buffer)
1) Autostainer Classical, Link 48.
2) BenchMark GX, XT, Ultra, Ultra Plus
3) Bond III, Prime, Max

Ready-To-Use antibodies and corresponding systems
mAb clone **VE1**, product no. **760-5095**, Ventana/Roche: Protocols with optimal results were typically based on HIER using CC1 as retrieval buffer (efficient HIER time 48-64 min.), 16-32 min. incubation of the primary Ab and OptiView (760-700) with or without OptiView Amplification kit (760-099) as detection system. Using these protocol settings, 72/78 (92%) laboratories produced a sufficient staining result (optimal or good).

Table 3 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU system. The performance was evaluated both as "true" plug-and-play system performed strictly according to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the intended IHC stainer device are included.

Table 3. **Proportion of sufficient and optimal results for BRAF for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
mAb clone **VE1**, **760-5095**	97% (32/33)	70% (23/33)	89% (103/116)	63% (73/116)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Significant modifications: retrieval method, retrieval duration and Ab incubation time altered, detection kit – only protocols performed on the specified vendor IHC stainer are integrated.

Comments
In this second NordiQC assessment of BRAF, a pass rate of 81% was obtained.

The prevalent feature of an insufficient result of BRAF V600E mutation was characterized by false positive reaction and/or an aberrant nuclear staining or poor-signal to noise ratio, seen in 88% of the insufficient results (36 of 41). The false positive results were mainly seen in the epithelial cells of appendix, but also in the non-mutated tumours (see Figs. 1b and 4b). The remaining problems were caused by completely false negative or too weak staining reaction (n=5).
The majority of all laboratories were able to demonstrate BRAF V600E mutation in the malignant melanoma with verified BRAF V600E mutation, tissue core no. 3, but only antibodies and protocols with high level of analytical sensitivity managed to demonstrate BRAF V600E mutation in the colon adenocarcinoma, tissue core no. 5, with verified V600E mutation.

18% (40 of 211) of the laboratories used Abs as Conc. format within a LD assay for BRAF mutation. Sufficient and optimal staining results were only obtained with the mAb clones **VE1** and **IHC600**.
Both the Ventana Benchmark platforms (Ventana/Roche) and Dako Omnis (Dako/Agilent) were widely used for staining with the VE1 clone as a concentrate with good results.

On the Ventana Benchmark platforms (Ventana/Roche), 91% (10 of 11) of the protocols produced sufficient results. Nine laboratories using OptiView with amplification achieved a strong signal in the two mutated neoplastic tissues (core no. 3 and 5). It was found critical not to increase the primary antibody incubation time beyond 32 minutes, as this caused unwanted granular cytoplasmic staining and aberrant nuclear reactions in the non-mutated tissues, interfering with the interpretation. Similar it was observed that extending amplification time could also lead to false positive results in the appendix epithelium. An extensive positive cytoplasmic staining reaction in the appendix was downgraded when observed (see Fig. 1b). Typically the mean titer of mAb clone VE1 as concentrate used was 1:700 with HIER in an alkaline buffer, CC1 for 64 min.
Appling the mAb clone VE1 on the Dako Omnis (Dako/Agilent) as a conc. format 73% produced a sufficient result (8 of 11). All laboratories used the FLEX+ system incorporating mouse linker to the protocol. Two laboratories used the FLEX++ system adding dual linker to the protocol, this did however not benefit the assay causing an increased background reaction with one case even producing a false positive result. This pattern was also observed when applying the Ab in a too high concentration, below 1:200. The staining pattern of the mutated neoplastic tissues generally exhibited weak to moderate intensity when using HIER in alkaline buffer for 30-60 minutes. Increasing the HIER time to 60 minutes (n=5) instead of 30 minutes enhanced the staining intensity, although it also negatively impacted the tissue morphology.
At this point it has to be emphasized that using traditional 3-step multimer/polymer detections system as OptiView (Ventana/Roche) or FLEX+ (Dako/Agilent) the BRAF V600E mutated neoplasias showed a weak to moderate, but unequivocal cytoplasmic staining reaction, where as using OptiView with amplification kit (tyramide based), the staining intensity was significantly increased. However, both levels of intensity was considered as optimal providing the staining reaction could easily be interpreted.

5 laboratories applied the mAb clone VE1 on the Leica Bond platforms and none producing an optimal result. In general the results were marked with comments on either poor signal-to-noise ratio or false positive results. All laboratories used the Ab in a concentration of 1:100 which for both the Ventana and Dako platforms were a too high concentration. It was also noted that the laboratories applied a relatively short HIER (20 min.) and a long incubation of the primary antibody (30-60 min.). These settings typically produced an inferior outcome on both Ventana and Omnis platforms and could be the reason for the lesser outcome on the Leica Biosystem Bond platforms, however the combination of no optimal results identified and the relatively limited number of observations challenges the conclusions.

The rmAb clones **RM8** and **ZR6** were used by 4 laboratories all with insufficient results. The staining pattern for the two clones were very similar with only a faint staining of the mutated neoplastic tissues but a moderate to strong reaction in the normal epithelium of the appendix and with a strong staining in serum.

81% (171 of 211) of the laboratories used Abs in RTU formats.

The most widely used RTU system for BRAF, **Ventana/Roche 760-5095** was based on mAb clone **VE1**, being used by 74% of all participants with intended use on the BenchMark systems and provided a pass rate of 97% (70% optimal) if using the vendor recommended protocol settings and 89% (63% optimal) if modifying the protocol (see Table 3). The majority of insufficient staining results were typically associated with an increased analytical sensitivity of the protocols causing a false positive staining result in the epithelial cells of the appendix. This reaction was accepted if only faint but downgraded when extensive and due to the interpretation challenges being induced questioning the specificity of the BRAF IHC assay, when non-mutated cells/tissues show an extensive staining reaction. The protocols giving an aberrant false positive cytoplasmic result was typically based on OptiView with amplification kit. Recently Ventana/Roche distributed a costumer notification letter informing that certain lots of OptiView provide a cross-reaction with a mucin-like protein in epithelial cells of e.g. appendix inducing a false positive result. This observation was indicated in the latest NordiQC assessment of ALK, run 73. This cross-reaction might be the explanation for the less successful application of OptiView with amplification kit, as the amplification kit will "amplify" both the specific signal and any unspecific target analyte identified by either the primary or secondary antibodies applied within the assay.
The protocol settings recommended by the vendor resulted in a weak to moderate reaction in the neoplastic tissues with mutations, and no staining was observed in any other tissue within the constructed TMA. The most widely used modification was related to an increased analytical sensitivity by either prolonging primary Ab incubation time and/or applying OptiView with amplification. 71 laboratories added amplification to the protocol providing a pass rate of 83% (59 of 71), 60% being optimal (43 of 71). This pass rate was largely similar to the one provided by vendor recommendations. However, protocols based on OptiView Amplification kit facilitated the interpretation as the positive cytoplasmic staining reaction in

the two neoplasias with BRAF V600E mutation was enhanced and still leaving the non-BRAF V600E mutated tumours negative.
It is well-known from previously NordiQC assessments, that assays based on OptiView Amplification kit (tyramide based) can be challenging to calibrate and frequently will provide a binary result as either negative or positive and not giving a "normal" dynamic range of antigen expression levels from low to high. The "lack" of dynamic range and the binary pattern can compromise the demonstration of low-level antigen expressing structures and at the same time also induce a risk of aberrant granular precipitation of the chromogen in structures expected to be negative.
This problem was also observed in this assessment, and it has to be emphasized that modifications of vendor clinically validated assays must be meticulously re-validated by the end-users on a large cohort of relevant tissue samples including attention to scoring guidelines for positive and negative read-out. The most successful application of OptiView with amplification was using amplification for 4+4 min. with long HIER (64 min.) and an Ab incubation time below 28 min.
The **VE1 Ventana/Roche 760-5095** system was used in 7 laboratories on unintended platforms, primarily Leica Bond platforms (n=6). The pass rate was 67%, however none optimal. The best results were provided by a long HIER time e.g. 60 min. and a short primary Ab incubation (15-20 min.).

Other systems as seen in Table 1c also provided optimal results, however the number of participants using these products were very limited.

In this second assessment of BRAF V600E the pass rate increased from 72% to 81%. The highest proportion of optimal results were provided by the RTU system from Ventana/Roche which provided optimal results both with vendor recommended protocol settings, but also with laboratory modifications when the analytical sensitivity was increased. In general, it seemed crucial to perform a long HIER to provide a strong enough signal to distinguish between a true weak positive staining result and a negative staining result.
Protocols based on the concentrated format of the mAb clone VE1 could provide optimal results on both the Ventana Benchmark platforms (Ventana/Roche) and the Dako systems (Dako/Agilent) but was challenged on the Bond systems (Leica Biosystems) giving no optimal results by the protocols submitted. In total 15 laboratoried used the mAb clone VE1 either as concentrate or RTU format on the Bond platform and an overall pass rate of only 33% (5 of 15) was observed.The main cause of insufficient results was caused by poor signal-to-noise ratio and in 40% of the cases also with an aberrant nuclear reaction.
It is hard to elude upon whether the problems lie within the detection system not being sensitive enough, and/or the laboratories not having a possibility to increase overall analytical sensitivity otherwise without causing an unwanted background reaction or if the protocols applied needed a more careful calibration adjusting the titre of the primary Ab in combination with other modifications as HIER and Ab incubation time.

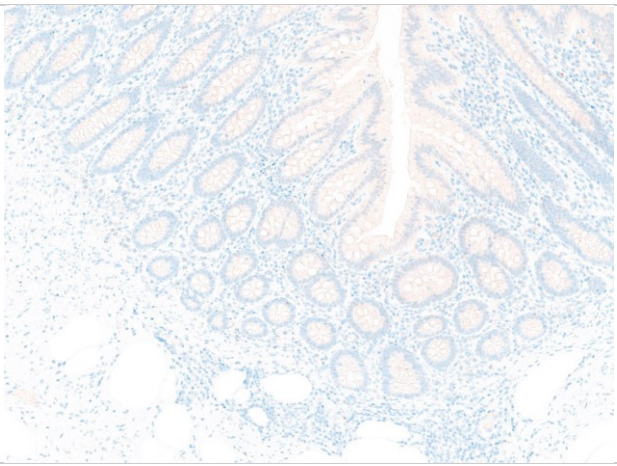


Fig. 1a (x100)
Optimal BRAF staining reaction of the epithelial cells in appendix using the Ventana RTU format 760-5095 based on mAb clone VE1, using OptiView with amplification (760-099) as detection kit, HIER for 64 min. and Ab incubation for 24 min. on Ventana BenchMark Ultra. No staining reaction is seen. Same protocol used in Figs. 2a-4a.

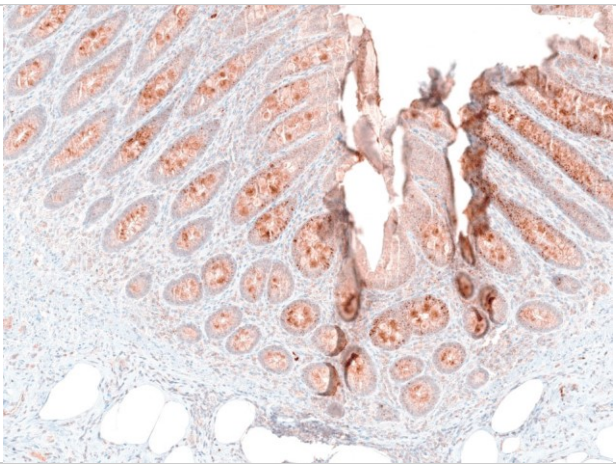


Fig. 1b (x100)
Insufficient BRAF staining reaction of the epithelial cells in appendix using the Ventana RTU format 760-5095 based on mAb clone VE1, using a modified protocol with OptiView and amplification (760-099) very similar to the protocol in figs. 1-4a but with a too long incubation of the primary Ab. Excessive background staining is seen along with an extensive false positive cytoplasmic staining reaction in the epithelial cells. Compare with optimal results in Fig. 1a. same field. Same protocol used in Figs. 2b-4b.

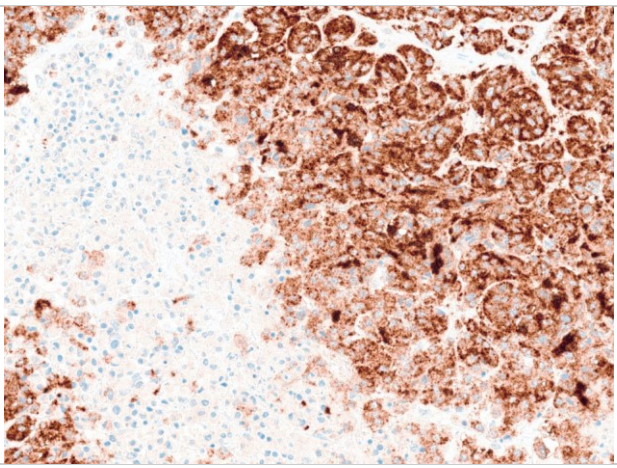


Fig. 2a (x200)
Optimal BRAF staining reaction of the malignant melanoma with BRAF V600E mutation, tissue core no. 3, using same protocol as in Fig. 1a. The neoplastic cells display a strong cytoplasmic staining reaction.

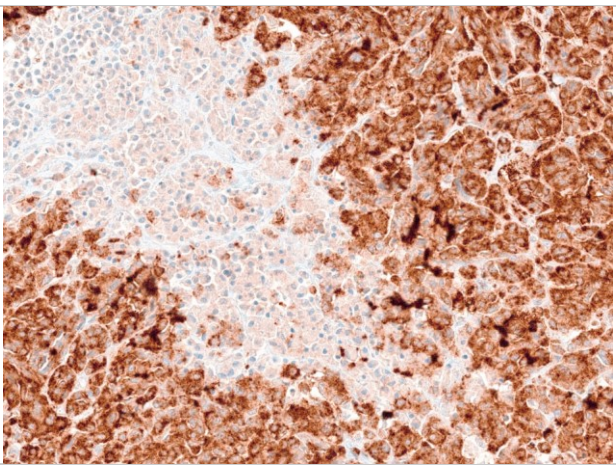


Fig. 2b (x200)
BRAF staining reaction of the malignant melanoma with BRAF V600E mutation, tissue core no. 3, using same insufficient protocol as in Fig. 1b. The neoplastic cells display a strong cytoplasmic staining reaction but also background reaction is observed in the negative area. Compare to fig. 2a. same field.

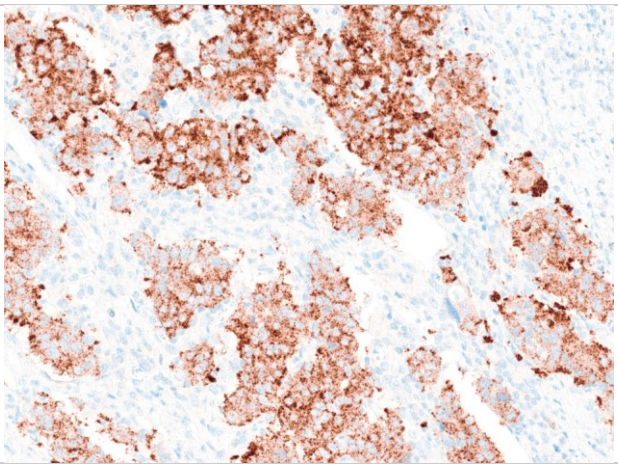


Fig. 3a (x200)
Optimal BRAF staining reaction of the colon adenocarcinoma with BRAF V600E mutation, tissue core no. 5, using same protocol as in Figs. 1a – 2a. The neoplastic cells display a moderate to strong cytoplasmic staining reaction.

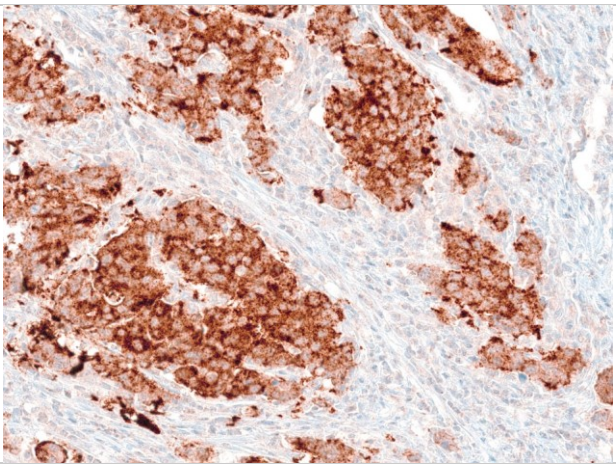


Fig. 3b (x200)
BRAF staining reaction of the colon adenocarcinoma with BRAF V600E mutation, tissue core no. 5, using same insufficient protocol as in Figs. 1b – 2b. The neoplastic cells display a strong staining reaction. A weak background reaction is seen in the stromal compartment. Compare with fig 3a. same field.

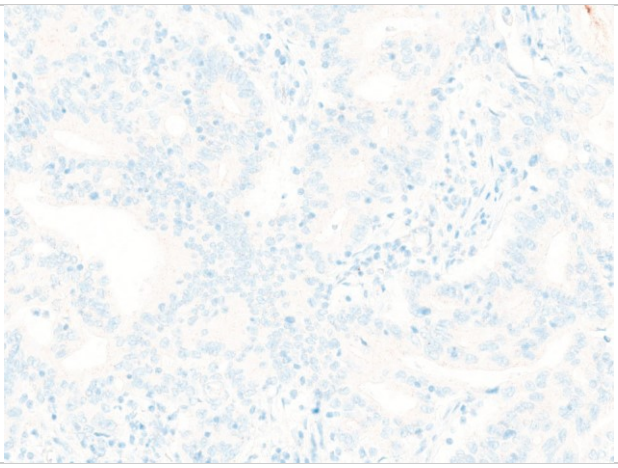


Fig. 4a (x200)
Optimal BRAF staining reaction of the colon adenocarcinoma without BRAF V600E mutation, tissue core no. 4, using same protocol as in Figs. 1a – 3a. All cells are negative.

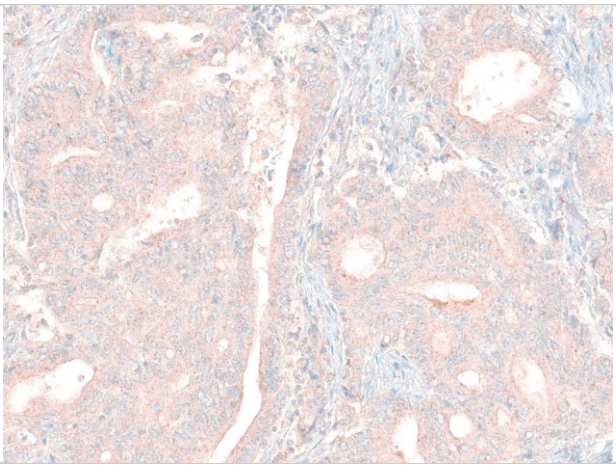


Fig. 4b (x200)
Insufficient BRAF staining reaction of the colon adenocarcinoma without BRAF V600E mutation, tissue core no. 4, using same protocol as in Figs. 1b – 3b. The neoplastic cells show a diffuse false positive staining reaction. Compare with optimal result in Fig. 4a.

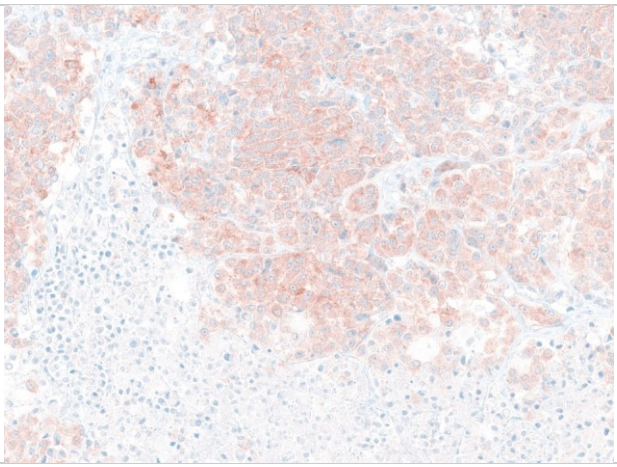
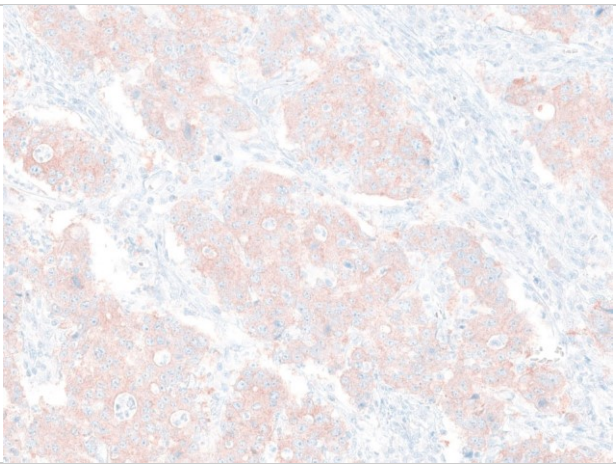
	

Fig. 5a (x200) Optimal BRAF staining reaction of the malignant melanoma with BRAF V600E mutation, using the Ventana RTU format 760-5095 based on mAb clone VE1, using the recommended protocol settings on Ventana BenchMark Ultra and a 3-step multimer based detection system The neoplastic cells display a weak to moderate but unequivocal cytoplasmic staining reaction. Also compare with Fig. 5b, same protocol and Fig 2a same field. Omission of amplification kit reduces the signal intensity significantly, but both levels are considered as optimal.	Fig. 5b (x200) Optimal BRAF staining reaction of the colon adenocarcinoma with BRAF V600E mutation, tissue core no. 5, using same protocol as in Fig 5a. The neoplastic cells display a weak but distinct and unequivocal cytoplasmic staining reaction. Also compare with Fig. 3a same field.

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